Abstract

The use of advanced imaging technologies for the identification of pancreatic cysts has become widespread. However, accurate differential diagnosis between mucinous cysts (MC) and nonmucinous cysts (NMC) consisting of pseudocysts (NMC1) and nonmucinous neoplastic cysts (NMC2) remains a challenge. Thus, it is necessary to develop novel biomarkers for the differential diagnosis of pancreatic cysts. An integrated proteomics approach yielded differentially expressed proteins in MC that were verified subsequently in 99 pancreatic cysts (21 NMC1, 41 NMC2, and 37 MC) using a method termed GeLC-stable isotope dilution-multiple reaction monitoring-mass spectrometry (GeLC-SID-MRM-MS) along with established immunoassay techniques. We identified 223 proteins by nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC/MS-MS). Nine candidate biomarkers were identified, including polymeric immunoglobulin receptor (PIGR), lipocalin 2 (LCN2), Fc fragment of IgG-binding protein (FCGBP), lithostathine-1-alpha (REG1A), afamin (AFM), chymotrypsin C (cathepsin C; CTRC), amylase, alpha 2B (pancreatic; AMY2B), lectin, galactoside-binding, soluble, 3 binding protein (LGALS3BP), and chymotrypsin-like elastase family, member 3A (CEL3A), which were established as biomarker candidates for MC. In particular, we have shown that a biomarker subset, including AFM, REG1A, PIGR, and LCN2, could differentiate MC not only from NMC (including NMC1) but also from NMC2. Overall, the MS-based comprehensive proteomics approach used in this study established a novel set of candidate biomarkers that address a gap in efforts to distinguish early pancreatic lesions at a time when more successful therapeutic interventions may be possible. Cancer Res; 75(16); 3227–35. ©2015 AACR.

Introduction

Recently, the use of ultrasound (US), multidetector computed tomography (CT), and magnetic resonance imaging (MRI) techniques have been most commonly used for the detection of pancreatic cysts. The prevalence of pancreatic cysts within the population ranges from 2.6% to as high as 44.7% worldwide (1–4), and a study from Japan reported that 24.3% of the population has pancreatic cysts based on autopsy cases. However, an accurate differential diagnosis of cystic pancreatic lesions remains a challenge.

Accurate differential diagnosis of mucinous cysts (MC) from nonmucinous cysts (NMC), including pseudocysts (NMC1) and nonmucinous neoplastic cysts (NMC2), is extremely important, because MC has malignant potential and requires surgical resection. Usually NMC1 are easily differentiated from MC because of their typical history and imaging characteristics, but sometimes these cysts are difficult to differentiate without histology.

Although endoscopic ultrasound (EUS), EUS-guided fine needle aspiration (EUS-FNA), and cyst fluid analysis all provide additional information for the differential diagnosis of pancreatic cysts in clinical practice, the diagnostic accuracy of these techniques is not satisfactory. The diagnostic accuracy of CT or MRI ranges from 55% to 75% depending on the reviewers. The accuracy of EUS morphology, cytology, and cystic carcinoembryogenic antigen (CEA) ranges from 50% to 75% (5).

The successful completion of human genome projects has led to the development of various proteomics-based approaches used to identify novel potential biomarkers that are capable of predicting diseases at early stages with minimal invasiveness (6–10). In fact, recent technical advances in mass spectrometry (MS) have been able to detect a large number of low-abundance proteins in biologic specimens, which results in the ability to identify many protein surrogates that may indicate malignant properties. As such, this is an attractive method to easily monitor global health and disease development.
proteome alterations to identify meaningful protein differences among disease states. Identifying proteomic differences in pancreatic cysts may provide differences between malignant tumors and other controls due to its proximity to the disease site (11).

Currently, the analysis of cyst fluid CEA is considered the best discriminating tool for mucinous and nonmucinous lesions of the pancreas; however, obtaining sufficient amounts of cyst fluid is not easy for accurate CEA analysis. Furthermore, the utility of cyst fluid CEA has been limited due to its insufficient sensitivity or specificity. On the other hand, a proteomic analysis would require only small amounts of cyst fluid for testing, which would be a distinct advantage. Several proteins have been suggested as potential biomarkers to differentiate potentially malignant MC from NMC (11–15). Previous work on the global proteome of pancreatic cysts using mass spectrometry has discovered novel biomarkers and several biomarker candidates (e.g., olfactomedin-4, mucin-18, and solubilized CEA-related cell adhesion molecules) that were differentially expressed in malignant lesions. To verify these biomarker candidates, IHC or enzyme-linked immunosorbent assays were performed. However, most of the biomarker candidates identified could not be sufficiently validated as different between patients and healthy donors. Antibody-based approaches have generally been used to verify an identified novel biomarker, but several limitations of these platforms should be carefully considered, including (i) the large amount of sample needed in cases where a large number of required proteins screens, (ii) antibody problems in developing a suitable experimental design, and (iii) the difficulty of obtaining high quality antibodies.

Therefore, in this study, we performed a MS-based approach to overcome these obstacles. We identified potential proteomic marker candidates discriminating MC from other pancreatic cysts by differential proteomics analysis. For biomarker validation, a GeLC-stable isothe dilution-multiple reaction monitoring-mass spectrometry (GeLC-SID-MRM-MS), which is a very sensitive and specific proteomics-based quantitative method, allowed for the detection of protein at low attomole concentrations with a minimal amount of specimen consumed without the need for the use of antibodies.

Sample preparation for proteomics experiments
A MARS column (Agilent Technologies) was used to remove six abundant proteins (albumin, transferring, IgG, IgA, anti-trypsin, and haptoglobin) from pancreatic cyst fluid while all other proteins were enriched. Seventy micrograms of depleted cyst fluid was resolved on 4% to 12% NuPAGE gels to reduce the complexity. Briefly, Coomassie blue–stained protein lanes on the SDS–PAGE gel were manually cut into 18 bands and subjected to in-gel tryptic digestion prior to liquid chromatography/tandem mass spectrometry (LC/MS-MS).

For GeLC-SID-MRM-MS experiments, cyst fluid was fractionated on SDS–PAGE and stained with Coomassie blue and then cut into three bands followed by in-gel trypsin digestion at a 50:1 ratio for 16 hours at 37°C. The tryptic digests were recovered by extraction with 50% acetonitrile (ACN)/0.1% formic acid and were dried. Synthetic peptides containing stable-isotope labels were spiked in tryptic digests of cyst fluid as standards (100 fmol/µl of each peptide). These mixtures were purified using an OMIX C18 tip (Agilent Technologies). Samples were dried and reconstituted with 10 µl 0.1% trifluoroacetic acid in water.

Mass spectrometry-based experiments
For the proteomic profiling of pancreatic cyst fluids, MS analysis of cyst fluid tryptic digests was performed using a 6520 quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies). The Q-TOF LC/MS was operated at high resolution (4 GHz) on positive ion mode for all experiments. All data were acquired in the data-dependent MS/MS mode, where a full MS scan was followed by three MS/MS scans for the three most abundant precursor ions in the MS survey scan.

For the determination of the cystic proteome of interest, we achieved MRM using a QTRAP 5500 (AB Sciex) equipped with a nano-electrospray ion source. An MRM scan was performed on the positive mode with ion spray voltages in a 2,200 to 2,500 V range.

Proteomic data acquisition
All MS/MS spectra were searched against the human Swiss-Prot database using the Spectrum Mill search engine (rev A.03.03.084 SR4, Agilent Technologies). Search results were automatically validated using Spectrum Mill software (Agilent Technologies) to meet a false discovery rate (FDR) <1%. Peptides with a 1% FDR cutoff were used to identify proteins via automatic validation using the following parameters: scored percent intensity (SPI) >70% for matches with scores >13 for +1, >=11 for +2, and >13 for +3. Then, proteins were filtered on the basis of the following criteria: SPI >70% for matches with scores >6 for +1, +2 and 3+, rank 1–2 score threshold = 2, and protein score > 20. Label-free and relative semi-quantification by spectral counts, which are useful for quantifying protein abundance changes in shotgun proteomics, were performed for biomarker discovery. The ratio of spectral counts (Rsc-value; ref. 16), which is the log2 (protein ratio) measured from spectral counts, was used for statistical validation of the proteomic data acquired. The Rsc > 1 indicates a 2-fold change in group comparison was allowed for the first screen of the protein list.

MRM transitions generated by MRMPilot v. 2.0 (AB Sciex) were monitored for MRM experiments. At least three transitions from one proteotypic peptide were generated; two or three peptide charge states containing 8–25 amino acids, no posttranslational modification (PTM), and one missed cleavage were allowed.

Patients and Methods

Study subjects
The Institutional Review Board at Samsung Medical Center (Seoul, South Korea) approved this study. The patients were recruited prospectively from February 2008 to January 2015. Surgical candidates were directly consulted for surgery. Other patients, who were assumed to have benign cysts with thin walls of small size (less than 2.0 cm) or typical serous cyst adenomas on CT or EUS, underwent follow-up on a regular basis without EUS-FNA.

Enrolled patients with pancreatic cysts underwent EUS-FNA. A Hitachi-Aloka with sector scan transducer (5 MHz) was used for the linear endoscopic ultrasounds. Two specialized endoscopic ultrasonographers performed each ELUS-FNA. Aspirated pancreatic cysts were described in terms of location, size, viscosity, and fluid volume. Each sample was subjected to cytology, tumor marker identification, and chemical analysis. Pancreatic cyst fluids were harvested, frozen immediately, and stored at −70°C for further cytologic examination and proteomic analysis.

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Characteristic NMC1
Baseline clinical characteristics

Table 1.

between 2008 and 2012 at Samsung Medical Center. A shortage
assessed with the nonparametric Mann
in the concentration of proteomic markers between groups were
analyzed with parametric tests. Differences
between characteristics: (i) cystic fluid CEA > 192 ng/mL
(iv) EUS consistent with intraductal papillary mucinous
neoplasm (IPMN) or MCN; (iii) cytology consistent with MC.
NMC2 were diagnosed on histology or based on any two of the
following characteristics: (i) cystic fluid CEA <5 ng/mL; (ii) EUS
consistent with NMC; (iii) cytology consistent with NMC.
Pseudocysts were diagnosed on histology based on EUS consis-
tent with pseudocyst and a typical clinical history including
cyst resolution on follow-up.

CEAs were measured using a radioimmunoassay kit (RIA-
KEY, Shin Jin Medics Inc.). Histologic or cytologic diagnoses
were made in 44 patients, and clinical diagnoses were made in
56 patients.

Biomarker identification

The analytic strategies of biomarker development in pancreatic
cysts are shown in Fig. 1. We identified 223 proteins (Supple-
mentary Table S1) and performed semi-quantification by spectral
counts, which represent the abundance of each protein, with the
aim to identify differentially expressed proteins (DEP; Supple-
mentary Table S2). In this study, we paid attention to a set of
proteins that changed in MC with high malignant potential. Thus,
we chose proteins with Rsc > 1 value that indicates a 2-fold change
in each group compared with the malignant group. Finally, we
identified 62 DEPs among the disease groups (NMC1, NMC2,
MC, and malignancy).

Biomarker prioritization

We used MRM assay to prioritize a set of proteins of interest
derived from the biomarker identification using shotgun prote-
omics and select specific peptides from these markers suitable
for the MRM assay. A total of 1,021 MRM transitions against 62
DEPs were created and then MRM experiments were achieved
on target peptides screened through the first discovery process
using QTRAP 5500 MS. On the basis of this analysis, 33
proteins consequently satisfied the filtering criteria on pooled
pancreatic cyst samples. Briefly, a reproducible chromatograph-
retention time property and strong MS signal intensity were
used to make a final clinical diagnosis (Supplementary Fig. S1;
ref. 17). MC were diagnosed on histology or based on any two of
the following characteristics: (i) cystic fluid CEA > 192 ng/mL
(ii) EUS consistent with intraductal papillary mucinous
neoplasm (IPMN) or MCN; (iii) cytology consistent with MC.
NMC2 were diagnosed on histology or based on any two of the
following characteristics: (i) cystic fluid CEA <5 ng/mL; (ii) EUS
consistent with NMC; (iii) cytology consistent with NMC.
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pancreatic cyst samples. Briefly, a reproducible chromatograph-
retention time property and strong MS signal intensity were

Table 1. Baseline clinical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NMC (n = 38)</th>
<th>Validation cohort 1</th>
<th>NMC2 (n = 21)</th>
<th>MC (n = 19)</th>
<th>Validation cohort 2</th>
<th>NMC (n = 24)</th>
<th>NMC2 (n = 20)</th>
<th>MC (n = 19)</th>
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<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td>57 (20–74)</td>
<td>49 (23–67)</td>
<td>56 (38–76)</td>
<td>44 (25–52)</td>
<td>50 (32–65)</td>
<td>60 (33–85)</td>
<td>65 (3–9)</td>
</tr>
<tr>
<td>Sex, N</td>
<td></td>
<td>11 (6/15)</td>
<td>7/11</td>
<td>3/1</td>
<td>4/16</td>
<td>5/14</td>
<td></td>
<td>10/9</td>
</tr>
<tr>
<td>EUS findings</td>
<td></td>
<td>15/2</td>
<td>6/15</td>
<td>4/1</td>
<td>4/12</td>
<td>9/10</td>
<td></td>
<td>11 (1–70)</td>
</tr>
<tr>
<td>Size, mm</td>
<td></td>
<td>5.5 (3–15)</td>
<td>3.2 (2–10)</td>
<td>3.5 (2–12)</td>
<td>6.5 (3–9)</td>
<td>3.5 (2–4)</td>
<td>3.7 (2–7)</td>
<td>4.7 (7–9)</td>
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<td>Unilocular/multilocular</td>
<td>6/15</td>
<td>6/15</td>
<td></td>
<td>4/1</td>
<td>4/0</td>
<td>9/10</td>
<td></td>
<td>10/9</td>
</tr>
<tr>
<td>Location of cyst, N (%)</td>
<td></td>
<td></td>
<td></td>
<td>3 (scanty-40)</td>
<td>12 (8–20)</td>
<td>7 (1–15)</td>
<td>11 (1–70)</td>
<td>6.5 (31.6)</td>
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<tr>
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<td>8 (38.1)</td>
<td>6 (33.3)</td>
<td>0 (0)</td>
<td>10 (50)</td>
<td>6 (31.6)</td>
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<tr>
<td>Body and neck</td>
<td>3 (17.6)</td>
<td>8 (38.3)</td>
<td>3 (16.7)</td>
<td>1 (25)</td>
<td>5 (25)</td>
<td>6 (31.6)</td>
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<tr>
<td>Tail</td>
<td>8 (47.1)</td>
<td>3 (14.3)</td>
<td>9 (50.0)</td>
<td>3 (75)</td>
<td>5 (25)</td>
<td>7 (36.5)</td>
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<td>Peri-pancreas</td>
<td>4 (23.5)</td>
<td>2 (9.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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<tr>
<td>Diagnostic methods</td>
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<td></td>
<td>16</td>
<td>1</td>
<td>2</td>
<td>9</td>
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<td>Surgery</td>
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<td>2</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>9</td>
<td>4</td>
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<tr>
<td>Cytology</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>4</td>
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<td>16</td>
<td>0</td>
<td>3</td>
<td>15</td>
<td>6</td>
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considered as follows: high MS response (signal to noise ratio > 8) and high reproducibility (±20 s retention time tolerance of three transitions). A total of 33 protein levels were relatively quantified on 56 pancreatic cysts (17 NMC1, 21 NMC2, and 18 MC) using MRM-MS without IS for initial screening. A disease specificity and diagnostic performance of candidates were referred for the second triage, and specific biomarkers were selected using the Kruskal–Wallis test, Mann–Whitney test, and ROC analysis. After this assessment, 17 proteins were found to be significantly altered in MC compared with NMC1 or NMC2 (P < 0.05 and AUC > 0.7).

Several proteins found in the discovery stage of our study overlapped with a set of biomarker surrogates previously reported in pancreatic cysts (Supplementary Table S3). However, these proteins were not specific for mucinous cysts and were not measurable using MRM assay; thus, they were eliminated in a further validation phase.

Biomarker validation

Of the 17 proteins isolated, nine proteins (LGALS3BP, PIGR, LCN2, FCGBP, REG1A, AFM, CTRC, AMY2B, and CELA3A) were finally selected for the validation phase based on the preliminary results by MRM experiments with isotope-labeled peptides. Herein, we conducted SDS-PAGE prior to MRM experiments in order to reduce the complexity of cyst fluids and to increase the sensitivity for the target proteins of interest (named GeLC-SID-MRM-MS). Their concentrations were then measured (Table 2 and Fig. 2) in cyst fluids using GeLC-SID-MRM-MS. In the validation cohort 1, the concentration of the seven proteins (PIGR, LCN2, FCGBP, REG1A, CTRC, AMY2B, and CELA3A) was higher in MC than NMC2 by approximately 7-fold. Afamin (AFM) levels were significantly decreased by 6- to 8-fold in MC (41 amol/mL) compared with NMC1 (260.7 amol/mL) or NMC2 (340 amol/mL), respectively. Specifically, four proteins, AFM, FCGBP, REG1A, and AMY2B, were found to be significantly altered in MC compared with NMC (including NMC1 and NMC2; P < 0.05; Table 3; Fig. 2). Thus, we additionally validated four proteins in a separate validation cohort 2. These markers also demonstrated surprisingly good diagnostic performance for discriminating MC and NMC (P < 0.001) as follows: AUCAF = 0.839 (95% CI, 0.687–0.937), AUCLCN2 = 0.895 (95% CI, 0.754–0.970), AUCREG1A = 0.945 (95% CI, 0.821–0.992), and AUCAM = 0.887 (95% CI, 0.744–0.966). Here, a biomarker subset, including AFM, FCGBP, REG1A, and AMY2B, showed good discriminatory performance in not only the validation cohort 1, but also the validation cohort 2.

In addition to the results of SID-MRM-MS, we performed immunoassays to detect LCN2, which had previously been shown to be present at higher levels in MC. Figure 3 shows that LCN2 levels are increased in MC by both methods. The Spearman correlation coefficient was used to determine a correlation...
between SID-MRM-MS and immunoassays ($r = 0.499; P = 0.0014$).

**Evaluation of multiple biomarkers**

A total of nine proteins were finally selected that showed differential expression in cystic fluids relative to NMC1, NMC2, and MC. To enhance the diagnostic power, we assessed the diagnostic performance, such as sensitivity, specificity, accuracy, and AUC among the three groups. These proteomic markers had a wide range of approximately 44% to 100% and 47% to 95% for sensitivity and specificity, respectively (Supplementary Table S4 and Table 3). Of these, four markers (AFM, REG1A, LCN2, and PIGR) selected through the validation cohort 1 also significantly differentiated MC from NMC.

**Table 2.** MRM transitions of the representative peptides corresponding to nine proteins

<table>
<thead>
<tr>
<th>Description</th>
<th>Peptide sequence</th>
<th>Mass information</th>
<th>CE(eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>AIPVTQ'YL'K</td>
<td>Light (Q1/Q3)</td>
<td>Heavy (Q1/Q3)</td>
</tr>
<tr>
<td></td>
<td>2/y5 516.8/652.4</td>
<td>520.3/659.4</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>2/y6 516.8/751.4</td>
<td>520.3/758.5</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>2/y7 516.8/848.5</td>
<td>520.3/855.5</td>
<td>28</td>
</tr>
<tr>
<td>CTRC</td>
<td>VSAYIDW''NEK</td>
<td>2/y5 669.3/689.4</td>
<td>672.8/696.4</td>
</tr>
<tr>
<td></td>
<td>2/y7 669.3/979.5</td>
<td>672.8/924.5</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>2/y8 669.3/1080.5</td>
<td>672.8/1087.6</td>
<td>34</td>
</tr>
<tr>
<td>CELA3A</td>
<td>LANPVSL''TDK</td>
<td>2/y6 529.3/662.4</td>
<td>532.8/669.4</td>
</tr>
<tr>
<td></td>
<td>2/y7 529.3/759.4</td>
<td>532.8/766.4</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>2/y8 529.3/875.5</td>
<td>532.8/880.5</td>
<td>28</td>
</tr>
<tr>
<td>LGALS3BP</td>
<td>IDITLSS'^K</td>
<td>2/y6 488.3/634.4</td>
<td>491.3/640.4</td>
</tr>
<tr>
<td></td>
<td>2/y7 488.3/747.5</td>
<td>491.3/753.5</td>
<td>26</td>
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<tr>
<td></td>
<td>2/y8 488.3/862.5</td>
<td>491.3/868.5</td>
<td>26</td>
</tr>
<tr>
<td>REG1A</td>
<td>WHWSSGLV''SYK</td>
<td>2/y10 718.8/1113.6</td>
<td>721.9/1119.6</td>
</tr>
<tr>
<td></td>
<td>2/y8 718.8/840.5</td>
<td>721.9/846.5</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>2/y9 718.8/927.5</td>
<td>721.9/933.5</td>
<td>37</td>
</tr>
<tr>
<td>LCN2</td>
<td>WYYVLGN'AIN''R</td>
<td>2/y10 716.4/983.6</td>
<td>719.9/990.6</td>
</tr>
<tr>
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<td>2/y11 716.4/1082.7</td>
<td>719.9/1089.7</td>
<td>37</td>
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<td>2/y9 716.4/884.5</td>
<td>719.9/891.5</td>
<td>37</td>
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<tr>
<td>AMY2B</td>
<td>LTGLDLAL''EK</td>
<td>2/y7 593.4/801.5</td>
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<td></td>
<td>2/y8 593.4/914.6</td>
<td>596.9/921.6</td>
<td>27</td>
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<td></td>
<td>2/y9 593.4/971.6</td>
<td>596.9/975.6</td>
<td>27</td>
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<tr>
<td>PIGR</td>
<td>IIEGEPNL''K</td>
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<td>510.3/664.4</td>
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<td></td>
<td>2/y7 506.8/786.4</td>
<td>510.3/793.4</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>2/y8 506.8/899.5</td>
<td>510.3/906.5</td>
<td>27</td>
</tr>
</tbody>
</table>

**NOTE:** Endogenous peptides (light) and isotope-labeled peptides (heavy) were chosen to measure biomarkers of interest. ^*, represents an amino acid labeled with a heavy isotope, 13C15N. The transitions (Q1/Q3) were optimized using MRMPilot software.

![Figure 2.](https://example.com) Concentrations of nine biomarkers in cystic fluids from patients diagnosed with NMC1, NMC2, and MC.
In the validation cohort 2. Interestingly, PIGR and LCN2 were specific to discrimination of MC from NMC and NMC2, respectively. Thus, we generated biomarker panel 1 (AUC, 0.919) by combining AFM, REG1A, and LCN2 for NMC2 versus MC. AFM, REG1A, and PIGR were chosen for differentiating MC from NMC as panel 2 (AUC, 0.933; Fig. 4).

To evaluate the diagnostic performance of four markers, we calculated the diagnostic probability score (PS) based on the sum of the hit numbers of five proteins, including cystic CEA, over cutoff values ranging from 0 to 5. We described PS with (PS +) and without (PS −) a CEA cutoff in both validation cohorts and the cutoff PS value of 3 was adopted for differential diagnosis of MC. Using a PS + cutoff, 25 of 37 MC patients were diagnosed with 80% accuracy (Supplementary Table S5). Moreover, when a PS − cutoff was applied to both validation cohorts, the performance was enhanced, with 30 of 37 MC patients accurately diagnosed, representing 81% sensitivity, 90% specificity, and 86% accuracy. We strongly suggest that a $3 \leq \text{PS}$ is indicative of high MC diagnostic potential.

**Discussion**

Pancreatic cysts have a wide spectrum of disease states ranging from benign to malignant. Differential diagnosis between NMC and MC is crucial to determining the most appropriate treatment because MC has malignant potential. Cystic fluid CEA, EUS-FNA, CT, and MRI have been used to aid in determining the final diagnosis in clinical practice, but their diagnostic accuracies are not satisfactory. Cystic fluid cytology has low sensitivity due to scant cell numbers in samples. CEA, a known clinical tumor marker, is also often suggested as a differential diagnostic marker for MC (5, 18–20). However, the optimal cutoff value of cystic fluid CEA ranges widely, between 109.9 and 480 ng/ml depending on the study. Its diagnostic accuracy for differentiating MC and NMC ranges

<table>
<thead>
<tr>
<th>Marker (amol/μL)</th>
<th>Median (95% CI)</th>
<th>NMC (n = 38)</th>
<th>MC (n = 18)</th>
<th>P</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>261 (139–724)</td>
<td>241 (171–577)</td>
<td>41.0 (20–77)</td>
<td>0.000</td>
<td>0.825</td>
</tr>
<tr>
<td>CTRC</td>
<td>374 (207–177)</td>
<td>92 (44–115)</td>
<td>354 (95–904)</td>
<td>0.188</td>
<td>0.608</td>
</tr>
<tr>
<td>CELAS3A</td>
<td>253 (76–996)</td>
<td>31 (22–186)</td>
<td>287 (73–827)</td>
<td>0.362</td>
<td>0.617</td>
</tr>
<tr>
<td>LGALS5BP</td>
<td>94 (95–157)</td>
<td>44 (22–87)</td>
<td>41 (14–58)</td>
<td>0.149</td>
<td>0.635</td>
</tr>
<tr>
<td>FGGBP</td>
<td>1,149 (617–2,737)</td>
<td>223 (119–418)</td>
<td>1,273 (701–4,431)</td>
<td>0.021</td>
<td>0.696</td>
</tr>
<tr>
<td>REG1A</td>
<td>321 (51–688)</td>
<td>59 (39–153)</td>
<td>490 (348–1,007)</td>
<td>0.017</td>
<td>0.727</td>
</tr>
<tr>
<td>LCN2</td>
<td>1,414 (470–4,755)</td>
<td>218 (95–608)</td>
<td>1,717 (649–5,910)</td>
<td>0.067</td>
<td>0.719</td>
</tr>
<tr>
<td>AMY2B</td>
<td>1,680 (251–4,928)</td>
<td>88 (39–380)</td>
<td>1,304 (668–5,446)</td>
<td>0.074</td>
<td>0.652</td>
</tr>
<tr>
<td>PIGR</td>
<td>13 (9–45)</td>
<td>32.5 (11–93)</td>
<td>89 (29–609)</td>
<td>0.004</td>
<td>0.728</td>
</tr>
</tbody>
</table>

**Table 3.** A comparison of potential proteomic markers

NOTE: Statistical comparisons were made among the three groups of pancreatic cysts for the top nine markers [NMC (controls) vs. MC (cases) or NMC2 (controls) vs. MC (cases)]. A $P \leq 0.05$ was considered statistically significant.
from 72% to 86% in the literature. Although the primary aim of EUS-FNA is to determine malignancy, the diagnostic yield of EUS-FNA in this study was very disappointing (52.6%). In addition, CT or MRI images were included in the characterization of cystic pancreatic masses (55%–75%). Thus, high-quality diagnostic and prognostic tools are required allowing for minimally invasive testing with better accuracy.

For decades, various MS techniques have increased for diagnostic use in clinical laboratories for clinical application. Despite these efforts, MS-based approaches are being used more often to identify small molecules rather than protein markers. In fact, only a few proteomic investigations of pancreatic cyst fluids have been applied to overcome the current diagnostic limitations (11, 13, 15) for the differential diagnosis of cysts and early prediction of pancreatic cancer. Nevertheless, no reliable biomarkers have been discovered that can discriminate MC with malignant potential from NMC. Thus, our group focused on identifying proteomic markers that are differentially expressed in MC with high malignant potential compared with NMC, which would be helpful for the differential diagnosis of pancreatic cysts.

We analyzed pancreatic cyst fluids using integrated proteomics and described the differential expression profiles in different cystic lesions including NMC1, NMC2, and MC. Specially, nine proteins of interest (PIGR, LCN2, FCGBP, REG1A, AFM, CTRC, AMY2B, LGALS3BP, and CELA3A) were verified using MS-based targeted proteomics technologies and immunoassays. These markers have been previously described as potential biomarker candidates associated with multiple cancer types in other studies. For instance, AFM was reported as a potential biomarker for ovarian cancer (21) and breast cancer (22), where AFM may play a role in the progression of cancer. Plasma AFM concentrations were validated by Western blotting and immunoassays in patients with ovarian cancer. Serum REG1A concentrations were shown to be significantly overexpressed in patients with various pancreatic diseases, cirrhosis, and various cancers of the digestive system (23). Abnormal expression of PIGR was reported in hepatocellular carcinoma (24) and lung cancer (25). LCN2 has been proposed as an early screening marker for human primary breast cancer (26, 27), ovarian cancer (28), colorectal cancer, and pancreatic cancer (29–31). The exact mechanism for how these proteins correlate with pathogenesis has yet to be clearly understood, despite that these proteins have been suggested as potential biomarkers in various cancers.

Our data also showed that three proteins (REG1A, PIGR, and LCN2) were upregulated and AFM was downregulated in MC compared with NMC2. This was in agreement with previous reports that showed alteration of these protein levels depending on malignant potential. In particular, PIGR protein was consistently upregulated in MC in comparison with not only NMC1 but also NMC2. NMC1 are inflammatory cysts and are not difficult to differentiate from NMC2 because the clinical history and course are different. Some inflammatory markers could be also upregulated in MC. Therefore, useful markers of MC need to be altered discriminately in both NMC1 and NMC2.

As mentioned above, cross-sectional imaging (e.g., CT, MRI) or conventional tumor markers (e.g., CEA, CA 19-9, and CA 72-4) for the characterization of pancreatic cyst lesions have not provided effective diagnoses in previous studies. In contrast, our data show that the four marker candidates (AFM, REG1A, PIGR, and LCN2) and the combination of these proteins (panels 1 and 2) can differentiate MC from NMC groups. Furthermore, the multi-marker panel has greater discriminatory power for NMC versus MC (AUC, 0.933) than that of each single marker (AUC, <0.829) in the validation phase.

Our study demonstrates the usefulness of MS-based comprehensive proteomics methodologies for the discovery and validation of candidate biomarkers for differential diagnosis of pancreatic cysts. Multiple biomarkers (AFM, REG1A, PIGR, and LCN2) were identified and validated as potential markers for MC with high malignant potential. The multi-marker panel has greater discriminatory power for NMC versus MC (AUC, 0.933) than that of each single marker (AUC, <0.829) in the validation phase.
LCN2) have been identified, but their reliability and usefulness need to be confirmed in a clinical setting using independent patient cohorts.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Park, K.H. Lee, J.K. Lee, S.-Y. Lee
Development of methodology: J. Park, J.K. Lee
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.H. Lee, KT. Lee
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Park, H.S. Yun, S.-Y. Lee
Writing, review, and/or revision of the manuscript: J. Park, J.K. Lee, J.K. Lee, S.-Y. Lee

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References


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Jisook Park, Hwan Sic Yun, Kwang Hyuck Lee, et al.


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